Prebiotic supplementation of *in vitro* faecal fermentations inhibits proteolysis by gut bacteria and host diet shapes gut bacterial metabolism and response to intervention

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Abstract

Metabolism of protein by gut bacteria is potentially detrimental due to production of toxic metabolites, such as ammonia, amines, *p*-cresol, and indole. Consumption of prebiotic carbohydrates produces specific changes in the composition and/or activity of the microbiota that may confer benefits upon host wellbeing and health. Here, we have studied the impact of prebiotics on proteolysis within the gut *in vitro*.

Anaerobic stirred batch cultures were inoculated with omnivore (n=3) and vegetarian (n=3) faeces. Four protein sources (casein, meat, mycoprotein and soy protein) with and without supplementation by a oligofructose enriched-inulin. Bacterial counts, and concentrations of short chain fatty acids (SCFA), ammonia, phenol, indole, and *p*-cresol were monitored during

fermentation. Addition of the fructan prebiotic Synergy1 increased levels of bifidobacteria (p= 0.000019 and 0.000013 for omnivores and vegetarians respectively). Branched chain fatty acids (BCFA) were significantly lower in fermenters with vegetarians' faeces (p=0.004), reduced further by prebiotic treatment. Ammonia production was lower with Synergy1. Bacterial adaptation to different dietary protein sources was observed through different patterns of ammonia production between vegetarians and omnivores. In volunteer samples with high baseline levels of phenol, indole, *p*-cresol and skatole, Synergy1 fermentation led to a reduction of these compounds.

Importance: Dietary protein intake is high in Western populations which could result in potentially harmful metabolites in the gut from proteolysis. In an *in vitro* fermentation model, addition of prebiotics reduced the negative consequences of high protein levels. Supplementation with a prebiotic resulted in a reduction of proteolytic metabolites in the model. A difference was seen in protein fermentation between omnivore and vegetarian gut microbiotas: bacteria from vegetarian donors grew more on soy and Quorn™, than on meat and casein with reduced ammonia production. Bacteria from vegetarian donors produced less BCFA.

Introduction

Dietary protein levels in western European populations can be as high as 105g/d according to the Food and Agriculture Organization (1). However, the recommended dietary allowance (RDA) is 56g/d for men and 46g/d for women (2). This may result in high residual colonic nitrogen, with dietary protein having escaped digestion in the upper intestine entering the large gut where it can become a substrate for the colonic microbiota. Approximately 16g of

49 protein will be present in the colon following ingestion of 105g protein/day of which 8g are
50 endogenous and 8g are exogenous (3, 4). Among the endogenous proteins, there are 69.2%
51 bacterial proteins, 16.9% mucin, 7.65% enzymes, and 6.2% mucosal cells (5, 6).
52
53 Anaerobic metabolism of carbohydrate by gut bacteria produces short chain fatty acids
54 (SCFA), and gases from different pathways. Production of SCFA, mainly acetate, propionate,
55 and butyrate, in the lumen is generally believed to mediate health benefits such as
56 maintaining colonic epithelial cell function, regulate energy intake and satiety, controlling
57 inflammation, and defend pathogen invasion (7). Microbial breakdown of protein not only
58 generates SCFA and gases, however, but also ammonia, amines, indolic and phenolic
59 compounds, and branched chain fatty acids (BCFA) through the deamination and
60 decarboxylation of amino acids (8). Though evidence on humans is scarce, in studies in rats
61 and in *ex vivo* studies, ammonia at a physiologically relevant dose can harm colon barrier
62 function, shorten colonocyte lifespan, and is co-carcinogenic in rats (9-11). Hydrogen
63 sulphide can be produced from sulphur containing amino acids and is toxic to colonocytes,
64 damaging DNA and blocking utilisation of butyrate as an energy source (12-15). Metabolism
65 of tyrosine, phenylalanine and tryptophan produces phenol, indole, *p*-cresol and skatole
66 which are potential carcinogens; phenol and *p*-cresol can reduce intestinal epithelial barrier
67 function *in vitro* (10, 16, 17). BCFAs are generated from branched chain amino acids such as
68 valine, leucine, and iso-leucine which make them biomarkers for bacterial proteolysis,
69 however there are no human physiological roles for BCFAs known (18).
70
71 Thus, foods entering the colon can have a health impact on the host, possibly by changing gut
72 microbiota composition and activity. The International Agency for Research on Cancer (19),
73 an agency under the World Health Organization (WHO) published a press release in October

2015: where it classified red meat as "probably carcinogenic to humans", and processed meat as "carcinogenic to humans", with concerns over colorectal cancer (19). Some epidemiological studies found reduced risk of colorectal cancer (CRC) with high consumption of dietary fibre, while red meat and processed meat had a positive correlation with CRC (20-23). Animal protein intake was associated with increased inflammatory bowel disease (IBD) risk in two Japanese and French studies (24, 25).

Increased consumption of prebiotics, which can reach the colon resulting in specific changes in the composition and/or activity in the gastrointestinal microflora, may counter the negative effects of gut microbial proteolysis in persons ingesting high protein diets (26). Inulin-type fructans can resist hydrolytic enzymes in the human GI tract and are resistant to small intestinal absorption, subsequently they become a substrate source for the microbiota within the large intestine. The impact of inulin on the gut microbiome has been studied using *in vitro* and *in vivo* approaches (27-29). The aim of this study was to understand metabolism of gut bacteria proteolysis in the distal colon and how prebiotics can affect the proteolysis, therefore, to investigate the potential of consuming prebiotics to counteract the negative effect of having high protein diet.

Results

Bacterial Enumeration

Total bacteria and most microbial groups that were monitored in this study reached the highest number after 24 hours incubation. However lactobacilli, *Faecalibacterium prausnitzii* and *Roseburia* numbers only increased in the first 10 hours with lactobacilli numbers in particular declining after 10 hours. Bacterial populations from omnivores and vegetarians

responded differently to the proteins: faecal bacteria from omnivores had insignificant higher counts on meat and casein than on soy protein and Quorn™ extract, while faecal bacteria from vegetarians had higher counts on soy protein and Quorn™ extract (8.75±0.40 $\log_{10}$ CFU/ml) than meat and casein (8.38±0.47 $\log_{10}$ CFU/ml) (p=0.03).

The vegetarian microbiota had higher bifidobacteria and lactobacilli counts at the beginning compared to omnivore microbiota (Supplementary Tables 1 and 2).

In order to investigate proteolytic bacteria, independent *t* tests were performed to compare samples with protein addition (casein, meat, mycoprotein and soy protein) and controls at 24 and 48 hours (Figure 1 and 2). Though there are studies confirming that many *Bacteroides.* spp., are proteolytic (30), we found no significant changes in *Bacteroides.* spp. on protein substrates. *Clostridium coccoides*, *Eubacterium rectale* and *Clostridium cluster* XIVa and XIVb grew on protein substrates: bacteria from omnivore donors had higher counts comparing to the control group (p=0.055) while those from vegetarian donors were significantly higher (p<0.01). *Roseburia* number did not change with protein added. *Atopobium* cluster from both omnivore and vegetarian donors grew on protein substrates with statistical significance. Clostridial cluster IX populations in cultures inoculated with samples from vegetarian donors increased on the protein substrates significantly, while cultures with omnivore samples were not statistically different. Lower counts of clostridial cluster IX in vegetarian donors' controls could explain the difference. *Desulfovibrio* counts were significantly higher with protein from both omnivore and vegetarian donors. *Clostridium* clusters I and II also grew more on proteins however, growth only reached statistical significance with inocula from vegetarians.

To investigate how prebiotics may modify the microbiota, independent *t* tests were used to

compare cultures with prebiotics and without, after 24 and 48 hours fermentation (Figure 1 and 2). Synergy1 addition significantly boosted the growth of total bacteria, bacteroides, clostridial cluster IX, bifidobacteria, and lactobacilli with both omnivore and vegetarian inocula, with bifidobacteria displaying the highest growth on Synergy1. In cultures with vegetarian donor' samples, *Clostridium coccoides*, *Eubacterium rectale* and *Clostridium cluster* XIVa and XIVb, *Roseburia*, *Faecalibacterium prausnitzii*, and *Atopobium* also had significant higher count with prebiotics than without. There were no inhibitory effects of prebiotics found on any of bacterial groups monitored in this study.

Organic Acids

Most organic acids accumulated during fermentation and reached their highest concentrations at 24 or 48 hours fermentation, with the exception of lactate which transiently increased during the first 10 hours then gradually decreased to below 1mM at 48 hours. Branched amino acids such as leucine and isoleucine can be metabolised by faecal bacteria to produce BCFA indicating proteolytic fermentation. Omnivores had higher BCFA production (4.03±5.25mM) while vegetarians had little production (1.61±1.60mM) ($p=0.004$). For instance, while growing on casein, bacteria from omnivores produced 10.19±8.62 and 13.13±10.93 mM of isobutyrate and isovalerate respectively, while bacteria from vegetarians produced 2.03±2.16 and 3.52±3.29 mM of isobutyrate and isovalerate (Supplementary Table 1 and 2).

Comparing samples with protein and without at 24 and 48 hours, cultures inoculated with both omnivore and vegetarian donors had significantly higher concentrations of acetate, propionate, isobutyrate, butyrate, and isovalerate on protein (Figure 3 and 4). However, fermentation samples with prebiotics had significantly elevated concentration of acetate and succinate at 24 and 48 hours, and significantly more lactate at 6 and 10 hours (Figure 3 and

5).

Butyric acid production was low in this study and no changes were found in cultures with omnivores samples; this correlates with the lack of differences in populations of butyrate-producing bacteria (*Roseburia* and *Faecalibacterium prausnitzii*). In samples with vegetarian donors' inocula, butyrate producers (*Clostridium coccoides*, *Eubacterium rectale* and *Clostridium cluster* XIVa and XIVb, *Roseburia*, *Faecalibacterium prausnitzii*) had significantly higher counts, however, butyrate production was not significantly increased. Concentrations of BCFA were lower on prebiotics although without statistical significance. Variation in BCFA production between donors was seen in this study, therefore, two-way ANOVA on isovalerate and isobutyrate was used to examine the effect of both treatment and donor on production. A significant influence of donor on isobutyrate and isovalerate was found with six donors ($p<0.01$). Donor variation may indicate that a larger sample size is needed to observe the inhibitory effect of prebiotics on BCFA production. (Supplementary Table 3)

Volatile Organic Compounds

This study quantified four potentially detrimental volatile organic compounds (VOCs) which were indole, phenol, *p*-cresol and skatole. Production of these compounds varied with individual donor and the effect of prebiotics on VOCs production also varied according to donor diet. Production of VOCs, from highest to lowest, was indole, phenol, *p*-cresol and skatole in most cases. However, with soy protein, phenol production was higher than indole production. With all donors, comparing negative and positive controls, the production of volatile compounds was reduced by Synergy1. However, comparing cultures on protein+Synergy1 with cultures on the corresponding protein, production of indole, phenol,

173 *p*-cresol and skatole were inhibited by Synergy1 after 48 hours fermentation with inocula
174 from omnivore donor 1, omnivore donor 2 and vegetarian donor 1. These three donors
175 produced relatively high levels of phenol and indole on protein(292.20±521.76 µg/ml)
176 compared with others (28.92±23.61 µg/ml) (p=0.02). Fermentation models inoculated with
177 these high VOCs producers, Synergy1+protein models produced significantly less phenol and
178 indole (113.21±227.94 µg/ml) (p=0.046).
179 Protein source affected production of VOCs. According to this study, casein resulted in the
180 highest concentration of VOCs in five donors, this was probably because casein is high in
181 aromatic amino acids which are the main substrates for bacteria to produce phenolic and
182 indolic compounds. Omnivore donor 3 had low phenolic production from casein correlating
183 with this donor's low total bacterial count (Supplementary Table 4).

184 **Ammonia**

185

186 Ammonia is a major metabolite of protein fermentation by faecal bacteria. Ammonia
187 concentrations increased gradually during fermentation on all substrates together with the
188 negative control when compared to the positive control. Ammonia concentrations on
189 Synergy1, however, remained at low levels (17.55±4.53mM at 48 hours for omnivores and
190 25.47±4.55mM for vegetarians) compared to all protein treatments in this study. The
191 volunteer diet also influenced the selective fermentation of faecal substrates. With faecal
192 samples from omnivores, fermentation resulted in higher ammonia levels on casein and meat
193 extract, however, with faecal samples from vegetarians, soy protein and Quorn extract
194 produced more ammonia (Figure 6).

195

196 Fermentation on protein for 24 hours resulted in significantly higher concentrations of
197 ammonia compared to fermentation without protein using both omnivore and vegetarian

samples ($p<0.001$). Fermentation on prebiotics resulted in significantly lower concentrations of ammonia in cultures with omnivore donors' faecal bacteria (Table 1).

Discussion

Lactate production peaked at 10hours fermentation while other organic acids concentrations kept increasing. This coincided with counts of lactobacilli and was to be expected as lactate can be utilised by several bacteria to produce other SCFAs. Changes in propionic acid producing *Bacteroides* and *Clostridium cluster* IX populations were seen and propionic acid increased in vessels containing Synergy1 with the difference reaching significance with omnivore donors' samples ($p=0.006$). Succinate is an intermediate product for propionate production, the succinate pathway being widely present in bacteroides (31). The significantly higher levels of succinate in samples with Synergy1 could be associated with propionate production by bacteroides.

Faecal bacteria responded differently on various substrates in pH controlled stirred batch cultures. Total bacteria number from vegetarians were significantly more on soy protein and Quorn™ than meat and casein. Host dietary habits may explain a preference for different protein sources. Growth of proteolytic bacteria from the human gut supported this: *Clostridium coccoides* and *Eubacterium rectale* from omnivore microbiota and vegetarian microbiota grew on meat/casein and soy/Quorn™ respectively (Supplementary Tables 1 and 2). Ammonia concentrations also indicate that an omnivore microbiota and a vegetarian microbiota favour different protein sources based on their host diet. A possible reason is differences in amino acid composition among various proteins: bacteria that have adapted to the host diet can breakdown peptides, metabolise amino acids or utilise coupled Stickland amino acid fermentation.

By observing fermentation characteristics of the negative controls: saccharolytic bifidobacterial growth at 6 hours with omnivore faeces occurred, indicating that there was a small amount of undigested saccharides within the omnivore faecal sample. However, this was not seen from the vegetarian donors.

Even when total bacteria tend to be more saccharolytic, there were some proteolytic bacteria present in the gut microbiota. The genus *Clostridium* contains more than 100 species and these bacteria can be saccharolytic, proteolytic, or both. Within clostridial clusters I and II, there are saccharolytic species such as *C. butyricum* and *C. beijerinckii*; *C. sporogenes* and *C. acetobutylicum* are both saccharolytic and proteolytic; there are proteolytic species such as *C. limosum* and *C. histolyticum* (32). This might explain why *Clostridium* spp. grew on prebiotics with a vegetarian microbiota: saccharolytic types from this genus were likely to be stimulated by prebiotics. This would also imply that these faecal bacteria from vegetarians are more saccharolytic than clostridia from omnivore donors.

Vegetarian donor 1 had the highest production of phenolic and indolic compounds together with the highest *E. coli* population which correlate with the ability of *E. coli* to produce phenolic compounds (33). Indole and *p*-cresol are conjugated as indoxyl sulphate and *p*-cresol sulphate in the human body; before they are excreted via urine, they are toxic to human endothelial cells, can reflect the progression of chronic kidney diseases, and increase cardiovascular disease risk for such patients (34-37). Therefore, reduced production of indole and *p*-cresol can benefit human health in many ways.

Studies feeding rats with different protein sources did not find higher colonic toxicity of casein comparing with soybean, which is contrary to the phenol and *p*-cresol results in this

study (38, 39). Feeding red meat gave higher DNA damage than feeding casein in rats (40). Similar effects were found in human epidemiological research: dairy products were inversely correlated with colorectal cancer in Finnish men and New York University women; they speculated that this protective effect may result from other nutrients in the dairy products but not from macronutrients such as protein (41, 42). Mycoprotein is a relatively new protein source from the filamentous fungus *Fusarium venenatumsource* under the trade mark of Quorn™ (43). Quorn™ products contain all the essential amino acids, are low in fat and high in dietary fibre. However, in terms of protein fermentation by gut microbiota, Quorn™ was no different to other proteins.

The use of pH controlled stirred batch culture systems allowed rapid analysis of different protein fermentations by gut microbiota and the impact of prebiotics. This fermentation system is limited however: SCFAs would be absorbed from the human colon and the digesta supply would be continuous.

Some animal studies and human studies have revealed an inhibitory effect of proteolysis by prebiotics such as resistant starch, FOS, and XOS (44-49). These were investigated by analysing indolic/phenolic compounds, or nitrogen secretion in the urine and faeces. One of these studies also compared DNA damage with and without resistant starch in rat colonic cells, and found that the starch protected cells from DNA damage (46). One possible mechanism of decreased proteolytic fermentation in the presence of prebiotics is through the enhanced growth of saccharolytic bacteria requiring more amino acids for growth, reducing amino acid availability for proteolytic bacteria.

Differences between the gut microbiotas from vegetarian and omnivore donors are not clear with three donors, however, fermentation patterns on different substrates were seen in this

study such as the differences in BCFA, ammonia, and total bacteria. In terms of protein fermentation by faecal bacteria, based on the different ammonia production and bacteria growth response to different protein source: microbiota from vegetarian donors have adapted to vegetarian protein sources and can utilise these proteins more efficiently. In addition, in this study, lower BCFA production was found with vegetarians' gut bacteria; this could suggest that these donors had lower branched chain amino acids in their diet. Prebiotic supplementation lowered proteolytic metabolites more in cultures with omnivores' samples comparing to cultures with vegetarians' bacteria: vegetarian donors are more likely to be on a high fibre diet and may need a higher dose of Synergy1 to see a prebiotic effect (50).

Addition of Synergy1 at the beginning to 48 hour batch culture fermentation changed the microbiota to a more saccharolytic nature by stimulation of bifidobacteria and lactobacilli without a significant change of *Clostridium* and *E. coli*. Supplementation with Synergy1 also reduced the concentration of protein metabolites (ammonia with significance and BCFA but not reaching significance); in those donors with high production of VOCs, inhibition was also found with Synergy1. An inulin rich diet could be beneficial in individuals with high protein diet, however, this effective dose of inulin is relatively difficult to achieve, especially in people consuming a Western diet (51, 52). Therefore, adding fructan prebiotics could potentially reduce the negative consequences of ingesting high protein diets, although this would need to be demonstrated *in vivo.* EFSA have approved the use of chicory inulin at a dosage of 12g per day to maintain normal bowel function, however, the effective does of prebiotics to regulate bacterial proteolysis is unknown (53). In this study, 5g of inulin type fructans were effective *in vitro*, but production of metabolites such as phenol and indole was only inhibited in some of the donors. This needs to be validated *in vivo* and a higher dose might have a better inhibitory effect and cover more of the population. This study also

296 revealed the importance of host habitual diet on the metabolic function of human gut
297 microbiome. This infers that host diet shapes the gut bacteria in a profound way. The
298 individual difference is significant which again could due to individual diet difference.

299 ## Materials

300 ### Proteins

301 Protein substrates used were casein hydrolysate (Sigma-Aldrich, Poole, UK, meat extract for
302 microbiology (Sigma-Aldrich, Poole, UK), soy protein acid hydrolysate powder (Sigma-
303 Aldrich, Poole, UK), and mycoprotein which was extracted from a commercial product
304 (Quorn^{TM}) purchased from a local supermarket.

305 ### Prebiotic

306 Inulin-type fructan was a mixture of oligofructose and inulin: 50%±10% DP (degree of
307 polymerisation) 3-9 and 50%±10% DP≥10 (Orafti®Synergy 1, BENEO-Orafti, Tienen,
308 Belgium).

309 ## Methods

310 ### Protein extraction

311 Mycoproteins were extracted from Quorn^{TM} based on the method described by R. J. H.
312 Williams et al. (54). Quorn^{TM} mince (500g) was mixed with 1200ml water and then
313 homogenised in a blender. 60ml of formic acid was added after homogenisation and the pH
314 lowered to 1.6. Afterwards, 5g pepsin was added and the solution incubated at 37°C for 48
315 hours. Samples were centrifuged at 3000g for 15 minutes and the supernatants freeze-dried
316 for later use. After extraction, the nitrogen content of mycoproteins was quantified using the
317 Kjeldahl method (Campden BRI, UK) and was found to be 10.3%. The remaining

mycoprotein was stored at -20°C.

Protein dose determination

Based on previous validation work from *in vitro* batch culture experiments and in human trials, the dose of 1% of substrate (w/v) equates to 5g inulin reaching the colon (27, 55). Synergy1 (1% w/v) was used in this study to investigate the prebiotic effect. The approach used in a 150ml batch culture experiments to simulate high protein ingestion is shown in Table 2. The amount of casein, meat extract, mycoprotein and soy protein was adjusted based on their true protein content which is shown in Table 3.

In vitro batch culture fermentation

Faecal Sample Preparation

Ethical approval of collecting faecal samples from healthy volunteers was obtained from University of Reading University Research Ethics Committee in 2014. Faecal samples were obtained from three healthy meat eating individuals and three healthy vegetarian volunteers between the ages of 18 and 60 (vegetarians 34.44±6.03 years old and omnivores 29.33±3.06) who had not taken antibiotics for at least six months prior to the experiment and had no history of gastrointestinal disorders. None were taking prebiotic supplements. All volunteers were following their diet for at least 5 years.

Faecal samples were diluted 1 in 10 (w/v) using 1M, pH7.4, anaerobically prepared phosphate buffered saline (PBS, Oxoid, Hampshire, UK). This solution was homogenised in a stomacher (Seward, stomacher 80, Biomaster) for 120 seconds at normal speed. 15mL of this was then immediately used to inoculate batch culture vessels.

Batch Culture Basal Nutrient Medium.

Basal nutrient medium was prepared with chemicals obtained from Sigma-Aldrich, Poole, UK unless otherwise stated. In one litre: 2g peptone water, 2g yeast extract (Oxoid, Hampshire, UK), 0.1g NaCl, 0.04g K_2HPO_4 (BDH, Poole, UK), 0.04g KH_2PO_4 (BDH), 0.01g $MgSO_3.7H_2O$ (Fischer scientific, Loughborough, UK), 0.01g $CaCl_2.6H_2O$, 2g $NaHCO_3$ (Fischer), 0.5g L–cystine HCl, 2mL Tween 80, 10µL vitamin K1, 0.05g haemin, 0.05g bile salts (Oxoid), 4ml resazurin (pH7).

pH controlled, stirred batch culture fermentation

Vessels with an operating volume of 300mL were set up. 135mL of basal nutrient medium was autoclaved (121°C for 15 minutes) and aseptically poured into sterile vessels. This system was left overnight with oxygen free nitrogen sparging into the medium at a rate of 15mL/min. After 4 hours of fermentation, nitrogen flow was stopped and gas outlets were clamped to trap gas. pH meters (Electrolab pH controller, Tewksbury, UK) were connected to each vessel to regulate pH 6.7 to 6.9 with the aid of 0.5M HCl or NaOH.

Each vessel was also temperature controlled at 37°C and stirred using a magnetic stirrer. Prebiotic and relative protein treatment were added to the vessels prior to inoculation with 15mL of faecal inoculum. For each donor, 10 vessels were prepared for 10 treatments: casein, meat extract, Quorn, soy protein, casein+Synergy1, meat extract+Synergy1, Quorn+Synergy1, soy protein+Synergy1, Synergy1, and a negative control.

Samples were removed from the fermenters after 0, 6, 10, 24 and 48 hours incubation.

366 **Enumeration of faecal microbial populations by flow cytometry fluorescence *in situ***
367 **hybridisation (FISH)**
368
369 A 750µl sample of batch culture fluid was centrifuged at 11337 × g for 5 minutes and the
370 supernatant discarded. The pellet was then suspended in 375µl filtered 0.1M PBS solution.
371 Filtered cold (4°C) 4% paraformaldehyde (PFA) (1125µl) was added and samples were stored
372 at 4°C for 4 hours. These were then washed thoroughly with PBS to remove PFA and re-
373 suspended in a mixture containing 300 µl PBS and 300 µl 99% ethanol. Samples were then
374 stored at -20°C prior to FISH analysis by flow cytometry. Filtered cold (4°C) 0.1M PBS (500
375 µl) was mixed with fixed samples (75µl), before centrifuged at 11337 × g for 3 minutes. The
376 pellets were then resuspended in 100µl of TE-FISH (Tris/HCl 1 M pH 8, EDTA 0.5 M pH 8,
377 and filtered distilled water with the ratio of 1:1:8) containing lysozyme solution (1 mg/ml of
378 50,000 U/mg protein). Samples were then incubated in the dark at the room temperature for
379 10 minutes, and then centrifuged at 11337 × g for 3 minutes. Pellets were washed with 500µl
380 filtered cold PBS, and then washed with 150µl hybridisation buffer (5 M NaCl, 1 M Tris/HCl
381 pH 8, formamide, ddH2O, 10% SDS with the ratio of 180:20:300:499:1) and centrifuged at
382 11337 × g for 3 minutes. Pellets were then resuspended in 1ml of hybridisation buffer.
383 Aliquots (50µl) with 4µl of different probes (50 ng μl^{-1}) were incubated at 35°C for at least
384 10 hours. The probes used in this study are listed in Table 7. Non Eub, Eub338-I-II-III are
385 attached with fluorescence Alexa488 at the 5′ end, and other specific probes are attached with
386 Alexa647. A set of Non Eub, Eub338-I-II-III are attached with fluorescence Alexa647 at the
387 5′ end to be the controls. For samples to detect specific groups, 4µl of Eub338-I-II-III were
388 added together with 4µl specific probes. Hybridisation buffer (150µl) was added to each
389 aliquot after incubation, followed by 3 minutes centrifugation at 11337 × g. Supernatants
390 (150µl) were carefully removed before samples were centrifuged at 11337 × g for 3 minutes.

Remaining supernatant was then removed, and pellets were resuspended in 200µl washing buffer. Washing buffer was prepared as: 12.8µl of 5M Na Cl, 20µl of 1M Tris/HCl pH 8, 10µl of 0.5 M EDTA pH 8, and 1µl of 10 % SDS in 956.2µl of filtered cold distilled water. Samples were then incubated at 37°C for 20 minutes and centrifuged at 11337 × g for 3 minutes. After supernatant removal, pellets were resuspended in different volume of filtered cold PBS based on flow cytometry load. Bacteria counts were then calculated with the consideration of flow cytometry reading and PBS dilution.

Short chain fatty acid (SCFA) analysis by gas chromatography

Samples were centrifuged at 11337 × g for 10 minutes to remove all particulate matter. Supernatants were then filtered through a 0.2 µm polycarbonate syringe filter (VWR, Farlington, UK). Extraction was done with some modifications of a method from A. J. Richardson et al. (69). Filtered sample (500µl) was transferred into a labelled 100 mm×16 mm glass tube (International Scientific Supplies Ltd, Bradford, England) with 25 µl of 2-ethylbutyric acid (0.1 M, internal standard) (Sigma, Poole, UK). Concentrated HCl (250µl) and 1 ml diethyl ether were added to each glass tube and samples vortexed for 1 minute. Samples were then centrifuged at 2000 × g for 10 minutes. The diethyl ether (upper) layer of each sample was transferred to a labelled clean glass tube. A second extraction was conducted by adding another 0.5 ml diethyl ether, followed by vortexing and centrifugation. The diethyl ether layers were pooled. Pooled ether extract (400µl) and 50 µl N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) (Sigma-Aldrich, Poole, UK) were added into a GC screw-cap vial. Samples were left at room temperature for 72 hours to allow lactic acid in the samples to completely derivatise.

An Agilent/HP 6890 Gas Chromatograph (Hewlett Packard, UK) using an HP-5MS 30m×0.25mm column with a 0.25µm coating (Crosslinked (5%-phenyl)-methylpolysiloxane,

Hewlett Packard, UK) was used for analysis of SCFA. Temperatures of injector and detector were 275°C, with the column programmed from 63°C for 0 minutes to 190°C at 15°C min^{-1} and held at 190°C for 3 minutes. Helium was the carrier gas (flow rate 1.7 ml min^{-1}; head pressure 133 KPa). A split ratio of 100:1 was used. Quantification of the samples was obtained through calibration curves of lactic acid and acetic, propionic, butyric, valeric and branched SCFA (iso-butyric and iso-valeric) in concentrations between 12.5 and 100 mM.

Volatile organic compounds analysis by GC-MS

Entrapment of volatile compounds

All fermentation samples were adjusted to a pH of 7.0 ± 0.3 using hydrochloric acid or sodium chloride prior to volatile entrapment. Each sample (1 g) was placed in a 250mL conical flask fitted with a Dreschel head. The flask was placed in a water bath maintained at a temperature of 30°C for 1 hour. The flask was attached to oxygen-free nitrogen (40mL/min) which swept volatile compounds from the headspace above the sample onto a glass trap (4 mm i.d., 6.35 mm o.d. x 90 mm long), containing 85mg of Tenax TA poly (a porous polymer absorbent based on 2,6-diphenylene-oxide) (Supelco, Poole, UK). Following volatile extraction, 1µL of 1, 2 dichlorobenzene in methanol (653ng/µL) was added to each trap as an internal standard and the trap was then flushed with oxygen free nitrogen to remove moisture (100mL/min) for 10 minutes.

Gas Chromatography and Mass spectrometry (GC-MS)

Volatile compounds collected on the Tenax adsorbent were analysed using a Perkin-Elmer Claris 500 GC-MS, attached to an automated thermal desorber (Turbomatrix ATD, Perkin

440 Elmer, Beaconsfield, UK). Tenax traps were desorbed at 300°C for 10 min and the volatiles
441 cryofocused on the internal cold trap held at -30°C. After desorption, the cold trap was heated
442 to 300 °C at 40°C per second to release volatile material onto the GC column. GC separation
443 was carried out on a DB-5 non-polar column (60m x 0.32mm id, 1µm film thickness, J&W
444 Scientific from Agilent). Helium at 145 kPa was used as the carrier gas. The GC oven
445 temperature program was 2min at 40°C followed by an increase at 4°C/min up to 260°C,
446 where it was held for 10 min. All data were collected and stored using Turbomax software
447 (version 3.5, Perkin Elmer). Compounds were identified from their mass spectra and
448 identities confirmed by comparison of retention time (linear retention index, LRI) and mass
449 spectra with those of authentic compounds analysed in online library database. Analyses were
450 carried out using Agilent 6890/5975 GC-MS system (Agilent Technologies, Palo Alto, CA,
451 USA) fitted with a Turbomatrix ATD.
452 Indole, *p*-cresol and phenol (Sigma-Aldrich, Poole, UK) were diluted using the same internal
453 standard which was 1, 2 dichlorobenzene in methanol (653ng/µL). Quantification of the
454 samples was obtained through calibration curves of phenol, *p*-cresol, indole and skatole in
455 concentrations between 25 and 100 µg/ml.
456

457 **Ammonia Analysis**

458 Samples at 0, 10 and 24 hours were diluted 1 in 50 v/v prior to analysis. Ammonia
459 concentration of diluted fermentation samples was analysed using a FluoroSELECT™
460 ammonia kit (Sigma-Aldrich, Poole, UK). Reagent was prepared by combining 100 µL assay
461 buffer, 4 µL reagent A and 4 µL reagent B in the kit. 10 µL H_2O (blank) and 10 µL sample
462 was added to each glass vial. Afterwards, 100 µL reagent was added to each tube. Samples
463 were kept in the dark for 15 minutes at room temperature before they were read in the
464 fluorometer. Ammonia standards were prepared by diluting 20 mmol/L NH_4Cl in distilled

water and the concentration range was 0.25-1 mmol/L).

Statistical analysis

All statistical tests were performed with the use of IBM SPSS Statistics version 24 (IBM Corp, US). Results are presented as means ± SD. Changes in specific bacterial groups, organic acids, and ammonia were assessed among different treatments and time points using two-way ANOVA. Significant differences were assessed by *post hoc* Tukey HSD test. In addition, to monitor the influence of protein and prebiotics independent *t* tests were used for all variables. For branched chain fatty acid and ammonia, two-way ANOVA was used to assess treatment effect and donor difference.

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We declare that there is no conflict of interest.

Footnotes

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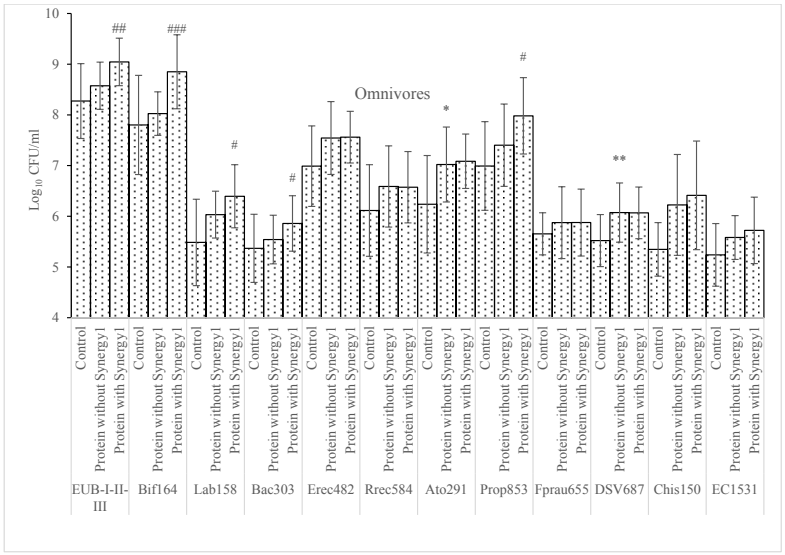
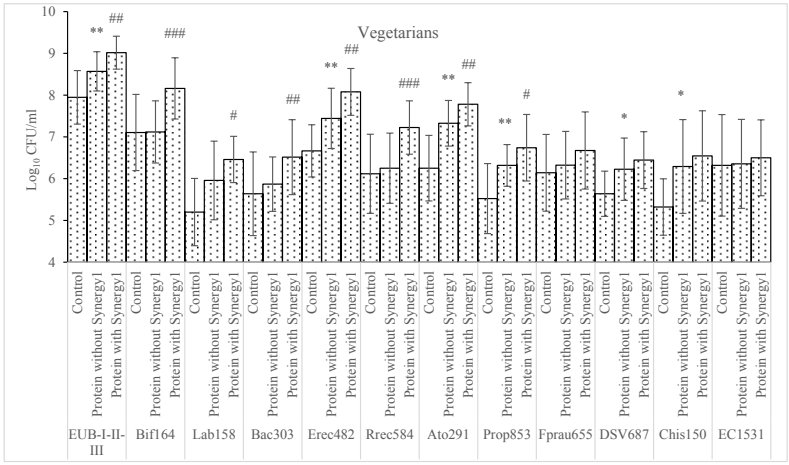
Figures



646

647 **Figure 1** Bacterial counts as $\log_{10}$ CFU/mL in the single stage batch culture as analysed by FISH. Values are mean values at 24 and 48 hours
648 fermentation from 3 omnivores' microbiota ± standard deviation. a: * Mean values were significantly different between control and protein
649 without Synergy1 ($p<0.05$). ** Mean values were significantly different between control and protein without Synergy1 ($p<0.01$). b: # Mean

values were significantly different between protein with and without Synergy1 ($p<0.05$). ## Mean values were significantly different between protein with and without Synergy1 ($p<0.01$). ### Mean values were significantly different between protein with and without Synergy1 ($p<0.001$).



656 **Figure 2** Bacterial counts as $\log_{10}$ CFU/mL in the single stage batch culture as analysed by FISH. Values are mean values at 24 and 48 hours
657 fermentation from 3 vegetarians' microbiota ± standard deviation. * Mean values were significantly different between control and protein
658 without Synergy1 ($p<0.05$). ** Mean values were significantly different between control and protein without Synergy1 ($p<0.01$). # Mean values
659 were significantly different between protein with and without Synergy1 ($p<0.05$). ## Mean values were significantly different between protein
660 with and without Synergy1 ($p<0.01$). ### Mean values were significantly different between protein with and without Synergy1 ($p<0.001$).
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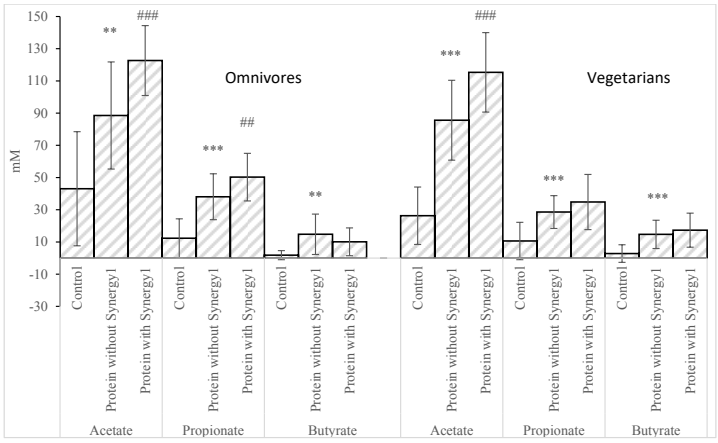

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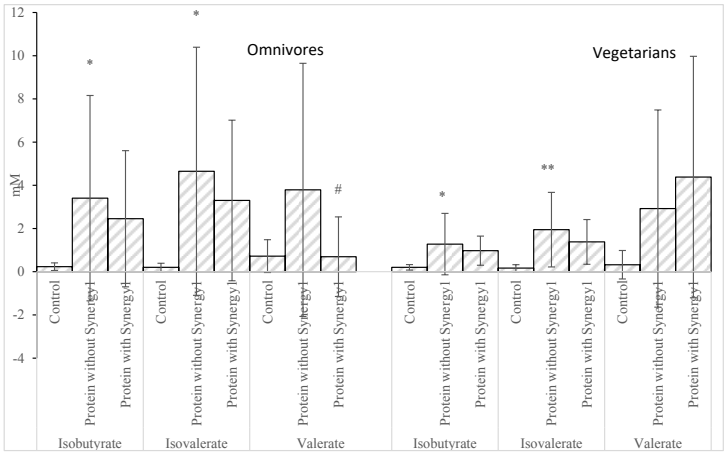
668 **Figure 3** SCFA differences between sample with and without protein as mM in the single stage batch culture. Values are mean values at 24 and
669 48 hours fermentation from 3 omnivores' microbiota and 3 vegetarians' microbiota ± standard deviation. * Mean values were significantly
670 different between control and protein without Synergy1 ($p<0.05$). ** Mean values were significantly different between control and protein
671 without Synergy1 ($p<0.01$). *** Mean values were significantly different between control and protein without Synergy1 ($p<0.001$). # Mean
672 values were significantly different between protein with and without Synergy1 ($p<0.05$). ## Mean values were significantly different between
673 protein with and without Synergy1 ($p<0.01$). ### Mean values were significantly different between protein with and without Synergy1

674 ($p<0.001$).



675

676 **Figure 4** BCFA and valerate differences between sample with and without protein as mM in the single stage batch culture. Values are mean
677 values at 24 and 48 hours fermentation from 3 omnivores' microbiota and 3 vegetarians' microbiota ± standard deviation. * Mean values were
678 significantly different between control and protein without Synergy1 ($p<0.05$). ** Mean values were significantly different between control and
679 protein without Synergy1 ($p<0.01$). *** Mean values were significantly different between control and protein without Synergy1 ($p<0.001$). #
680 Mean values were significantly different between protein with and without Synergy1 ($p<0.05$). ## Mean values were significantly different

between protein with and without Synergy1 ($p<0.01$). ### Mean values were significantly different between protein with and without Synergy1 ($p<0.001$).

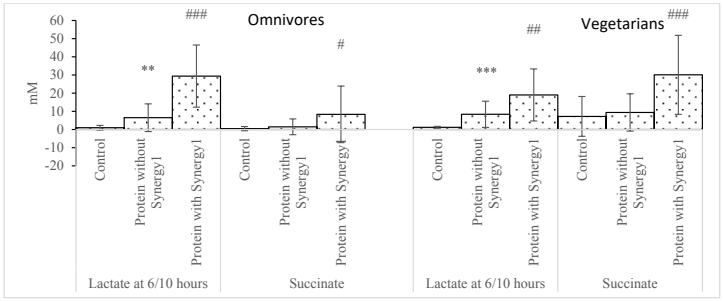


Figure 5 Lactate and succinate differences between sample with and without protein as mM in the single stage batch culture. Values are mean values at 24 and 48 hours fermentation unless specified from 3 omnivores' microbiota and 3 vegetarians' microbiota ± standard deviation. a: * Mean values were significantly different between control and protein without Synergy1 ($p<0.05$). ** Mean values were significantly different between control and protein without Synergy1 ($p<0.01$). *** Mean values were significantly different between control and protein without Synergy1 ($p<0.001$). b: # Mean values were significantly different between protein with and without Synergy1 ($p<0.05$). ## Mean values were significantly different between protein with and without Synergy1 ($p<0.01$). ### Mean values were significantly different between protein with and without Synergy1 ($p<0.001$).

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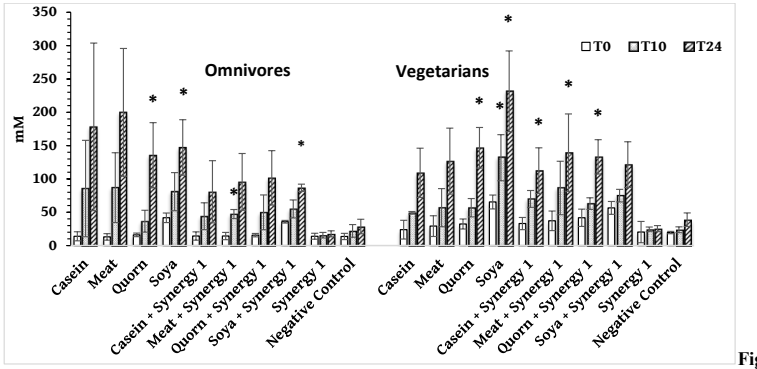

695 **Figure 6** Changes in ammonia concentration

696 (mM) of batch culture sample over time. Values are mean values at three time points from 3 omnivore and 3 vegetarian faecal donor's ± standard

697 deviation. * Mean values were significantly different from 0 hour fermentation samples ($p<0.05$).

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	Omnivores			Vegetarians		
	Control	Protein without Synergy1[a]	Protein with Synergy1[b]	Control	Protein without Synergy1[a]	Protein with Synergy1[b]
	n=6	n=12	n=12	n=6	n=12	n=12

Ammonia in mM	23.07±9.58	165.24±77.44***	91.16±33.24**	32.02±8.97	153.53±62.69***	126.64±35.76

Table 1 Ammonia concentration in samples as mM in the single stage batch culture. Values are mean values at 24 hours fermentation from 3 omnivores' microbiota and 3 vegetarians' microbiota ± standard deviation. a: *** Mean values were significantly different between control and protein without Synergy1 ($p<0.001$). b: ** Mean values were significantly different between protein with and without Synergy1 ($p<0.01$).

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	In vitro fermentation dosage
Dietary protein	2.4g
Mucin	0.57g
Digestive enzymes	0.18g

Note: digestive enzyme is a mixture of 0.107g pepsin, 0.022g pancreatin, and 0.00079g α-amylase based on an *in vitro* upper gut digestion simulation paper (56)

726 **Table 2** Endogenous and exogenous protein dosage to simulate the *in vivo* effect of 105g dietary protein per day consumption for 150ml batch
727 culture experiment.

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Protein	Protein content	Protein dose
Casein	68.75%	3.5g
Soy protein	75%	3.2g
Meat extract	76%	3.2g
Mycoprotein	64.2%	3.7g

745 **Table 3** Protein dose that is equivalent to 2.4g dietary protein responding with protein content

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748 **Table 4** Name, sequence, and target group of oligonucleotide probes used in this study for FISH of bacterial enumeration

Probe name	Sequence (5' to 3')	Target groups	References
Non Eub	ACTCCTACGGGAGGCAGC	Control probe complementary to EUB338	(57)
Eub338	GCTGCCTCCCGTAGGAGT	Most Bacteria	(58)
Eub338II	GCAGCCACCCGTAGGTGT	Planctomycetales	(59)
Eub338III	GCTGCCACCCGTAGGTGT	Verrucomicrobiales	(59)

Bif164	CATCCGGCATTACCACCC	*Bifidobacterium* spp.	(60)
Lab158	GGTATTAGCAYCTGTTTC CA	*Lactobacillus* and *Enterococcus*	(61)
Bac303	CCAATGTGGGGGACCTT	Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae	(62)
Erec482	GCTTCTTAGTCARGTACC G	Most of the *Clostridium coccoides-Eubacterium rectale* group (Clostridium cluster XIVa and XIVb)	(63)
Rrec584	TCAGACTTGCCGYACCG C	*Roseburia* genus	(64)
Ato291	GGTCGGTCTCTCAACCC	*Atopobium* cluster	(65)
Prop853	ATTGCGTTAACTCCGGC AC	Clostridial cluster IX	(64)
Fprau65	CGCCTACCTCTGCACTAC	*Feacalibacterium prausnitzii* and relatives	(66)

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DSV687	TACGGATTTCACTCCT	*Desulfovibrio* genus	(67)
Chis150	TTATGCGGTATTAATCTY CCTTT	Most of the *Clostridium histolyticum* group (*Clostridium* cluster I and II)	(63)
EC 1531	CAC CGT AGT GCC TCG TCA TCA	*Escherichia coli* BJ4	(68)

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